



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 16/00, C12P 21/08, A61K 39/395</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/45450</b> <b>(43) International Publication Date:</b> 4 December 1997 (04.12.97)
<b>(21) International Application Number:</b> PCT/US97/09610 <b>(22) International Filing Date:</b> 30 May 1997 (30.05.97)  <b>(30) Priority Data:</b> 08/655,953                      31 May 1996 (31.05.96)                      US  <b>(71) Applicant:</b> HEALTH RESEARCH INC. [US/US]; Elm and Carlton Streets, Buffalo, NY 14263 (US).  <b>(72) Inventor:</b> SEON, Ben, K.; 204 Ranch Trail West, Williamsville, NY 14221 (US).  <b>(74) Agents:</b> NELSON, M., Bud et al.; Hodgson, Russ, Andrews, Woods & Goodyear, 1800 One M & T Plaza, Buffalo, NY 14203-2391 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ANTI-ENDOGLIN MONOCLONAL ANTIBODIES AND THEIR USE IN ANTIANGIOGENIC THERAPY  <b>(57) Abstract</b> <p>A monoclonal antibody, or fragments thereof, having binding specificity for both endoglin expressed on proliferating human vascular endothelial cells and on proliferating murine endothelial cells. Antiangiogenic therapy in mammals can be effected by administering to the mammalian host a therapeutically effective amount of an anti-endoglin monoclonal antibody, or fragments thereof, which is conjugated to at least one angiogenesis inhibitor or antitumor agent. The composition is useful for treating tumor and angiogenesis-associated diseases.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## ANTI-ENDOGLIN MONOCLONAL ANTIBODIES AND THEIR USE IN ANTIANGIOGENIC THERAPY

This invention was made with government support under grant CA 19304 awarded by the U.S. Public Health Service. The government has certain rights in the  
5 invention.

### FIELD OF THE INVENTION

The present invention is related to compositions and methods for antiangiogenic therapy of  
10 certain disease types in humans. More particularly, the present invention is related to the production of anti-endoglin monoclonal antibodies, and a method for treating cancers and other pathological conditions associated with angiogenesis in humans in conjunction  
15 with administration of immunoconjugates comprising anti-endoglin monoclonal antibodies.

### BACKGROUND OF THE INVENTION

#### 1. Endoglin

20 Endoglin is a homodimeric membrane glycoprotein which is expressed at high levels in proliferating vascular endothelial cells (Burrows et al., 1995, *Clin. Cancer Res.* 1:1623-1634). Thus, endoglin is primarily a proliferation-associated marker  
25 for endothelial cells undergoing active angiogenesis. However, there is some expression of endoglin by the vascular endothelium of normal tissues (Burrows et al., *supra*; Wang et al., 1993, *Int. J. Cancer* 54:363-370). Recently, human endoglin was determined to specifically  
30 bind transforming growth factor- $\beta$  (TGF- $\beta$ ), and the deduced amino acid sequence of endoglin showed strong homology to  $\beta$ -glycan, a type of TGF- $\beta$  receptor.

Murine endoglin has been characterized as a dimer with molecular size of approximately 180  
35 kilodaltons (kD). Human endoglin exists in two forms; i.e., a smaller 160 kD form and a larger 170 kD form with the difference between the two being found in the

- 2 -

cytoplasmic portion of the protein. Endoglin has an extracellular region, a hydrophobic transmembrane region, and a cytoplasmic tail. A comparison of the nucleotide sequence of human endoglin with murine  
5 endoglin reveals an identity of about 71 to 72% (St. Jacques et al., 1994, *Endocrinol.* 134:2645-2657; Ge et al., 1994, *Gene* 158:2645-2657). However, in the human and murine sequences encoding the transmembrane regions and cytoplasmic regions of endoglin, there is a 93-95%  
10 identity. Thus, in the human and murine sequences encoding the extracellular region, there is significantly less identity.

## 2. Monoclonal antibodies to endoglin

15 There have been several anti-endoglin monoclonal antibodies ("MAB") previously reported in the art. MAb SN6 is an antibody generated from immunization of mice with cell membranes of human leukemia cells (Haruta and Seon, 1986, *Proc. Natl. Acad. Sci.* 83:7898-  
20 7902). It is a murine MAB that recognizes human endoglin. MAb 44G4 is an antibody generated from immunization of mice with whole cell suspensions of human pre-B leukemia cells (Gougos and Letarte, 1988, *J. Immunol.* 141:1925-1933; 1990, *J. Biol. Chem.* 265:8361-  
25 8364). It is a murine MAB that recognizes human endoglin. MAb MJ7/18 is an antibody generated from immunization of rats with inflamed mouse skins (Ge and Butcher, 1994, *supra*). It is a MAB that recognizes murine endoglin. MAb Tec-11 is an antibody generated  
30 from immunization of mice with human umbilical vein endothelial cells (Burrows et al., 1995, *Clin. Cancer Res.* 1:1623-1634). It is a murine MAB with reactivity restricted to human endoglin.

By the use of anti-endoglin antibodies and  
35 various staining procedures known in the art, it has

- 3 -

been determined that endoglin is expressed at moderate to high levels in endothelial cells contained in:

- (a) human tumor cells such as from human leukemia, including non-T-cell-type (non-T) acute lymphoblastic leukemia (ALL), myelomonocytic leukemia; and tumor-associated vasculatures from human solid tumors, including angiosarcoma, breast carcinoma, cecum carcinoma, colon carcinoma, Hodgkins lymphoma, lymphoma, lung carcinoma, melanoma, osteosarcoma, ovarian carcinoma, parotid tumor, pharyngeal carcinoma, rectosigmoid carcinoma;
- (b) human vasculature from placenta, adrenal and lymphoid tissues; and
- (c) murine stromal cells from intestine, stomach, heart and reproductive tissues.

A lesser degree (weak) endothelial cell staining for endoglin has been observed in a variety of normal human adult tissue sections from lymph node, tonsil, spleen, thymus, kidney, lung and liver. However, it was found that different anti-endoglin antibodies directed against different epitopes on endoglin showed the same selectivity for tumor vasculature (Burrows et al., 1995, supra).

Increased endoglin expression on vascular endothelial cells has also been reported in pathological conditions involving angiogenesis. Such angiogenesis-associated diseases include most types of human solid tumors, rheumatoid arthritis, stomach ulcers, and chronic inflammatory skin lesions (e.g., psoriasis, dermatitis; Westphal et al., 1993, *J. Invest. Dermatol.* 100:27-34).

### 3. Angiogenesis

Angiogenesis is the formation of new capillary blood vessels leading to neovascularization. Angiogenesis is a complex process which includes a

- 4 -

series of sequential steps including endothelial cell-mediated degradation of vascular basement membrane and interstitial matrices, migration of endothelial cells, proliferation of endothelial cells, and formation of capillary loops by endothelial cells. Solid tumors are angiogenesis dependent; i.e., as a small solid tumor reaches a critical diameter, for further growth it needs to elicit an angiogenic response in the surrounding normal tissue. The resultant neovascularization of the tumor is associated with more rapid growth, and local invasion. Further, an increase in angiogenesis is associated with an increase risk of metastasis. Therefore, antiangiogenic therapy to inhibit tumor angiogenesis would suppress or arrest tumor growth and its spread.

In normal physiological processes such as wound healing, angiogenesis is turned off once the process is completed. In contrast, tumor angiogenesis is not self-limiting. Further, in certain pathological (and nonmalignant) processes, angiogenesis is abnormally prolonged. Such angiogenesis-associated diseases include diabetic retinopathy, chronic inflammatory diseases including rheumatoid arthritis, dermatitis, and psoriasis. Antiangiogenic therapy would allow modulation in such angiogenesis-associated diseases having excessive vascularization.

#### 4. Antiangiogenic therapy and vascular targeting therapy of human solid tumors

The progressive growth of solid tumors beyond clinically occult sizes (e.g., a few mm<sup>3</sup>) requires the continuous formation of new blood vessels, a process known as tumor angiogenesis. Tumor growth and metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels to deliver nutrients and oxygen for the tumor

itself to grow. Therefore, either prevention of tumor angiogenesis (antiangiogenic therapy) or selective destruction of tumor's existing blood vessels (vascular targeting therapy) present a strategy directed to preventing or treating solid tumors.

Since a local network of new capillary blood vessels provide routes through which the primary tumor may metastasize to other parts of the body, antiangiogenic therapy should be important in preventing establishment of small solid tumors or in preventing metastasis (See, e.g., Folkman, 1995, *Nature Medicine*, 1:27-31). On the other hand, the vascular targeting therapy which attacks the existing vasculature is likely to be most effective on large tumors where the vasculature is already compromised (See, e.g., Bicknell and Harris, 1992, *Semin. Cancer Biol.* 3:399-407). Monoclonal antibodies, and fragments thereof according to the present invention are used as a means of delivering to either existing tumor vasculature or newly forming tumor neovascularization therapeutic compounds in a method of antiangiogenic therapy.

#### 5. Mouse models for human disease

##### A. Athymic nude or SCID mouse model

In the following embodiments used to illustrate the invention, it is important to consider the following concept. The use of athymic nude mice with human tumor xenografts has been validated as a model for the evaluation of chemotherapeutic agents because the model has been shown to reflect the clinical effectiveness of chemotherapeutic agents in original patients treated with these agents; and reflects antitumor effects from the agents, such as tumor regression or inhibition of tumor growth, as consistent with the activity against the corresponding types of clinical cancer (See for

example, Neuwalt et al., 1985, *Cancer Res.* 45:2827-2833; Ovejera et al., 1978, *Annals of Clin. and Lab. Science* 8:50). SCID mice with human tumor xenografts has also been accepted by those skilled in the art as a model for the evaluation of chemotherapeutic agents.

Monoclonal antibodies are useful for selective delivery of anticancer agents to tumor target tissue(s). In that regard, anti-endoglin MAb's may be used to target human tumor vasculature. Athymic nude mice or SCID mice with human tumor xenografts is a model in which may be tested antibody-directed targeting of tumor vasculature in a process of antiangiogenic therapy. The problem, however, is that the neovascularization for human xenografts in the mouse model arises from the (mouse) host's tissues. Thus, the prior art anti-endoglin MAb's, which are restricted to reactivity with either human endoglin or murine endoglin, cannot be used in such mouse models to perform the studies necessary to evaluate the clinical efficacy, pharmacokinetics, and the possibility of adverse side effects of antiangiogenic therapy.

#### B. Mouse models for angiogenesis-associated diseases

In the following embodiments used to illustrate the invention, it is important to consider the following concept.

The use of mouse models of angiogenesis has been accepted and validated as a models for the evaluation of therapeutic agents because the models have been shown to reflect the clinical parameters characteristic of the respective disease, as well as predictive of the effectiveness of therapeutic agents in patients. These mouse models include, but are not limited to: mouse model for retinal neovascularization (Pierce et al., 1995, *Proc. Natl. Acad. Sci. USA* 92:905-909); mouse models for rheumatoid arthritis (MRL-lpr/lpr mouse



model, Folliard et al., 1992, *Agents Actions* 36:127-135; mev mouse, Kovarik et al., 1994, *J. Autoimmun.* 7:575-88); mouse models for angiogenesis (Majewski et al., 1994, *Int. J. Cancer* 57:81-85; Andrade et al., 1992, *Int. J. Exp. Pathol.*, 73:503-13; Sunderkotter et al., 1991, *Am. J. Pathol.* 138:931-939); mouse model for dermatitis (Maguire et al., 1982, *J. Invest. Dermatol.* 79:147-152); and mouse model for psoriasis (Blandon et al., 1985, *Arch. Dermatol. Res.* 277:121-125; Nagano et al., 1990, *Arch. Dermatol. Res.* 282:459-462). Thus, the prior art anti-endoglin MAb, which are restricted to reactivity with either human endoglin or murine endoglin, cannot be used in the mouse model for the respective angiogenesis-associated disease to perform the studies necessary to evaluate the clinical efficacy, pharmacokinetics, and adverse side effects of antiangiogenic therapy in humans.

Hence, a need still exists for anti-endoglin MAb which can be used in antiangiogenic therapy of human tumor angiogenesis, and of other human angiogenesis-associated diseases having excessive vascularization, which can be evaluated for clinical efficacy and pharmacokinetics in human xenograft-mouse models, or mouse models of angiogenesis-associated diseases.

#### SUMMARY OF THE INVENTION

Accordingly, it is a primary object of the present invention to provide anti-endoglin MAb which can be used in antiangiogenic therapy of human tumor angiogenesis.

It is another object of the present invention to provide anti-endoglin MAb which can be used in antiangiogenic therapy of human angiogenesis-associated diseases having excessive vascularization.

It is another object of the present invention to provide such anti-endoglin MAbs, which can be used in antiangiogenic therapy, and which can be evaluated in the athymic nude mouse model or mouse models of angiogenesis-associated diseases.

It is another object of the present invention to provide a method for antiangiogenic therapy of human tumor angiogenesis using anti-endoglin MAbs.

It is a further object of the present invention to provide a method for antiangiogenic therapy of human angiogenesis-associated diseases having excessive vascularization using anti-endoglin MAbs.

The foregoing objects are achieved by providing novel anti-endoglin MAbs reactive against human endoglin which are also, unexpectedly, crossreactive with murine endoglin. The MAbs of the present invention may be conjugated to antitumor agents in providing a method for antiangiogenic therapy of human tumor vasculature. The MAbs of the present invention may be conjugated to angiogenesis inhibitors in providing a method for antiangiogenic therapy of human angiogenesis-associated diseases having excessive vascularization. These and further features and advantages of the invention will be better understood from the description of the preferred embodiments when considered in relation to the figures in which:

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph depicting the cytotoxic activity against proliferating murine endothelial cells (SVEC4-10) of immunoconjugates comprising the anti-endoglin MAbs of the present invention and ricin A (RA).

FIG. 1B is a graph depicting the cytotoxic activity against proliferating murine endothelial cells (SVEC4-10) of immunoconjugates comprising the anti-endoglin

MAbs of the present invention and deglycosylated ricin A (dgRA).

FIG. 2A is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7).

FIG. 2B is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following treatment with unconjugated MAb K4-2C10.

FIG. 2C is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following treatment with an immunoconjugate (K4-2C10-dgRA conjugate).

FIG. 2D is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following pretreatment with unconjugated anti-endoglin MAb (K4-2C10) and then subsequent administration of the immunoconjugate (K4-2C10-dgRA conjugate).

FIG. 3A is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7).

FIG. 3B is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following treatment with unconjugated MAb K4-2C10.

FIG. 3C is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following treatment with an immunoconjugate (K4-2C10-dgRA conjugate).

FIG. 3D is a graph depicting the progression in SCID mice of a human xenograft consisting of breast cancer cells (MCF-7), following pretreatment with unconjugated anti-endoglin MAb and then subsequent administration of the immunoconjugate (K4-2C10-dgRA conjugate).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The term "angiogenesis-associated disease" is used herein, for purposes of the specification and claims, to mean certain pathological processes in humans where angiogenesis is abnormally prolonged. Such angiogenesis-associated diseases include diabetic retinopathy, chronic inflammatory diseases, rheumatoid arthritis, dermatitis, psoriasis, stomach ulcers, and most types of human solid tumors.

The term "angiogenesis inhibitor" is used herein, for purposes of the specification and claims, to mean a biomolecule including, but not limited to, peptides, proteins, enzymes, polysaccharides, oligonucleotides, DNA, RNA, recombinant vectors, and drugs which function to inhibit angiogenesis.

Angiogenesis inhibitors are known in the art and include natural and synthetic bio-molecules such as paclitaxel, O-(chloroacetyl-carbonyl) fumagillol ("TNP-470" or "AGM 1470"), thrombospondin-1, thrombospondin-2, angiostatin, human chondrocyte-derived inhibitor of angiogenesis ("hCHIAMP"), cartilage-derived angiogenic inhibitor, platelet factor-4, gro-beta, human interferon-inducible protein 10 ("IP10"), interleukin 12, Ro 318220, tricyclodecan-9-yl xanthate ("D609"), irsogladine, 8,9-dihydroxy-7-methyl-benzo[b]quinolizinium bromide ("GPA 1734"), medroxyprogesterone, a combination of heparin and cortisone, glucosidase inhibitors, genistein, thalidomide, diaminoantraquinone, herbimycin, ursolic acid, and oleanolic acid.

The term "antiangiogenic therapy" is used herein, for purposes of the specification and claims, to mean therapy targeted to proliferating vasculature in which endoglin is expressed at high levels (as compared to quiescent vasculature); whether the therapy is directed against angiogenesis (i.e., the formation of

new capillary blood vessels leading to neovascularization), and/or existing vasculature which is proliferating and relating to a disease condition (e.g., vascular targeting therapy).

5           The term "antibody fragment" or "fragment thereof" is used herein, for purposes of the specification and claims, to mean a portion or fragment of an intact antibody molecule, wherein the fragment retains antigen-binding function; i.e., F(ab')<sub>2</sub>, Fab',  
10 Fab, Fv, Fd' and Fd fragments. Methods for producing the various fragments from MAbs are well known to those skilled in the art.

          The term "immunoconjugate" is used herein, for purposes of the specification and claims, to mean a  
15 conjugate comprised of the anti-endoglin MAbs or a fragment thereof according to the present invention, and at least one antitumor agent or at least one angiogenesis-inhibitor. Such antitumor agents are known in the art and include, but not limited to, toxins,  
20 drugs, enzymes, cytokines, radionuclides, photodynamic agents, and angiogenesis inhibitors. Toxins include ricin A chain, mutant *Pseudomonas* exotoxins, diphtheria toxoid, streptonigrin, boamycin, saporin, gelonin, and pokeweed antiviral protein. Drugs include daunorubicin  
25 and methotrexate. Radionuclides include radiometals. Cytokines include tumor necrosis factor. Photodynamic agents include porphyrins and their derivatives. The methods for complexing the anti-endoglin MAbs or a fragment thereof with at least one antitumor agent are  
30 well known to those skilled in the art (i.e., antibody conjugates as reviewed by Ghetie et al., 1994, *Pharmacol. Ther.* 63:209-34). Often such methods utilize one of several available heterobifunctional reagents used for coupling or linking molecules.

- 12 -

The term "mammalian host" or "host" is used herein, for purposes of the specification and claims, to mean a mouse or a human.

The term "monoclonal antibody" is used herein, for purposes of the specification and claims, to mean murine monoclonal antibodies and engineered antibody molecules made therefrom in which the anti-endoglin binding specificity for crossreactive epitopes between human endoglin and murine endoglin is maintained, such as chimeric or "humanized" antibodies, and as will be more apparent from the following embodiments. Hybridoma cell lines Y4-2F1, K4-2C10, P3-2G8, and D4-2G10, which produce monoclonal antibodies illustrative of the present invention, have been deposited with the American Type Culture Collection, and bear ATCC Designations HB-12171, HB-12172, HB-12173, and HB-12174, respectively.

The term "tumor" is used herein, for purposes of the specification and claims, to mean a tumor expressing endoglin at moderate to high levels (as compared to expression by normal tissue of the same type) such as human leukemias, including non-T-cell-type (non-T) acute lymphoblastic leukemia (ALL), myelomonocytic leukemia; and human solid tumors, with its surrounding vasculature expressing endoglin at moderate to high levels (as compared to expression by normal tissue of the same type) including angiosarcoma, breast carcinoma, cecum carcinoma, colon carcinoma, Hodgkins lymphoma, lymphoma, lung carcinoma, melanoma, osteosarcoma, ovarian carcinoma, parotid tumor, pharyngeal carcinoma, and rectosigmoid carcinoma.

A drawback to systemic therapies is the lack of selectively delivering the therapy to its intended target, diseased tissue, rather than to normal tissue. Monoclonal antibodies have been used to deliver therapeutics with greater target specificity, thereby

reducing toxicity. Murine MAbs or fragments thereof have been used to treat human disease, often with modest to substantial clinical efficacy (see, e.g., Ghetie et al., 1994, *supra*). Studies show that murine monoclonal antibodies may be repeatedly given on a safe basis, even if a human anti-mouse antibody response develops. Additionally, a human anti-mouse antibody response did not cause significant clinical problems with further repeated infusions of the murine MAb (see, e.g., Frodin et al., 1992, *Cell Biophys.* 21:153-165).

Targeting therapies against tumor vasculature, as opposed to the tumor itself, has several advantages. Typically, immunoconjugates are administered intravenously so that tumor vasculature can be saturated much quicker than saturation of tumor antigen. Therefore, a lesser amount of immunoconjugate is needed in a regimen, thereby decreasing the potential for non-specific side effects. Secondly, a substantial number of tumor cells are vitally dependent upon a small number of capillary loops. Thus, minimal damage to such capillary loops can result in significant and amplifying tumor cell death. Additionally, as described above, an anti-endoglin MAb conjugate can be used selectively against a variety of tumor types and other pathological conditions having excessive vascularization. While the anti-endoglin MAb targets antiangiogenic therapy to the tumor vasculature, it is appreciated by those skilled in the art that the delivered antitumor agent may additionally act directly on tumor cells with which it comes in contact.

One embodiment of the present invention comprises a novel class of murine MAbs characterized by their binding specificity for crossreactive epitopes between human endoglin and murine endoglin; stronger immunoreactivity with human endoglin as compared to murine endoglin; and selective immunoreactivity which is

restricted to certain tumor vasculature and proliferating vascular endothelial cells, as characteristic of angiogenesis. Thus, unlike the prior art anti-endoglin MAb which are restricted to reactivity with either human endoglin or murine endoglin, the anti-endoglin MABs of the present invention can be used in the athymic nude mouse model or other mouse models of angiogenesis-associated diseases to perform the studies necessary to evaluate clinical efficacy, pharmacokinetics, and potential adverse side effects of antiangiogenic therapy in humans. Four such anti-endoglin MABs have been developed to date: MABs K4-2C10, D4-2G10, Y4-2F1 and P3-2G8. A specific embodiment illustrating this novel class of the anti-endoglin MABs according to the present invention is MAB K4-2C10.

Another embodiment of the present invention comprises a method for using anti-endoglin MABs, or a fragment thereof, according to the present invention in antiangiogenic therapy. In this embodiment, an immunoconjugate is formed by coupling the anti-endoglin MABs, or a fragment thereof, to at least one antitumor agent, wherein the resultant immunoconjugate retains its selective immunoreactivity restricted to certain tumor vasculature and proliferating vascular endothelial cells characteristic of angiogenesis. The immunoconjugate is administered to an appropriate mouse model for testing the antiangiogenic therapy, with administration dependent upon the location of target vasculature. Having evaluated parameters such as clinical efficacy and pharmacokinetics in the appropriate mouse model, the antiangiogenic therapy may then be "scaled up" to human treatment. A physiological basis for scaling up the therapeutic agents comprising MABs from a mouse model to humans is known to those skilled in the art (see, e.g., Baxter et al., 1995, Cancer Res. 55:4611-4622, herein incorporated by reference).



**EXAMPLE 1**

## Production of crossreactive anti-endoglin MAb

At the time of the present invention, the previously reported anti-endoglin MAb were generated by immunizing mice with crude preparations containing endoglin, including cell membranes or whole cell suspensions of leukemia cells, inflamed skin, and human umbilical vein endothelial cells. The specificity of the anti-endoglin MAb so produced were reported to be either to human endoglin or to mouse endoglin. In contrast, the crossreactive anti-endoglin MAb of the present invention were generated by immunization of mice with purified human endoglin ("hEDG").

hEDG was purified from human acute lymphoblastic leukemia (ALL) cells. Cell membrane glycoproteins were isolated using detergent extraction and lectin affinity chromatography as described previously (Haruta and Seon, 1986, *Proc. Natl. Acad. Sci. USA*, 83:7898-7902, herein incorporated by reference). The isolated glycoproteins were applied to an immunoaffinity column containing anti-endoglin MAb SN6 (Haruta and Seon, 1986, *supra*) which had been equilibrated with 25 mM Tris-HCl, pH 8.0 containing 0.5% taurocholate, 0.15 M NaCl, 2 mM EDTA, 0.03% NaN<sub>3</sub>, and 0.5 mM phenylmethylsulfonyl fluoride. The bound materials were eluted with 50 mM diethylamine-HCl, pH 11.3 containing 0.5% taurocholate, 2 mM EDTA, 0.03% NaN<sub>3</sub>, ("alkaline buffer"). The eluted material was reapplied to the immunoaffinity column and the bound material was eluted with the alkaline buffer further containing 0.01% cytochrome-c (a 12.4 kD carrier protein) and neutralized. The eluted material was concentrated. This purification process was carried out at 4-6°C. Purification of hEDG was monitored by a solid phase radioimmunoassay using MAb SN6, and confirmed by gel electrophoresis with silver staining. The resultant

- 16 -

hEDG preparation contained a single major component of 170 kD under unreduced conditions, and 92 kD under reduced conditions.

In a first immunization protocol, 2 female  
5 BALB/c mice were immunized with the isolated hEDG. Briefly, an antigen solution comprising 10  $\mu$ g of the hEDG preparation in 100  $\mu$ l of 10 mM Tris-HCl buffer, pH 7.5, with 0.5% taurocholate, 0.15 M NaCl, and 14  $\mu$ g cytochrome-c, was mixed with an equal volume of adjuvant  
10 and then injected subcutaneously at multiple sites on each of the mice. In addition,  $1 \times 10^9$  *Bordetella pertussis* bacteria in 100  $\mu$ l saline were injected at different sites. Two booster immunizations of the antigen solution in adjuvant were administered. A last  
15 immunization comprising 40  $\mu$ l antigen solution containing 8  $\mu$ g hEDG preparation mixed with 200  $\mu$ l saline was administered intraperitoneally. The spleens were removed and fused with P3/NS1/1-Ag4-1 (NS-1) mouse myeloma cell line 4 days after the last immunization.  
20 Cell fusion, hybridoma screening, and immunoglobulin class determination were performed as described previously (Haruta and Seon, 1986, *supra*). In a second immunization protocol, a female BALB/c mouse was immunized with the isolated hEDG as described for the  
25 first experiment, but without the administration of *B. pertussis*. Eleven hybridomas generated by these immunizations produce individually different anti-hEDG MAbs that were further characterized.

30

## EXAMPLE 2

### Characterization of anti-endoglin MAbs

In this embodiment is illustrated the binding specificity of four of the eleven different anti-human endoglin antibodies generated using the methods  
35 according to Example 1. These four MAbs, K4-2C10, D4-2G10, Y4-2F1 and P3-2G8, which react with endoglin

expressed on human cells were found to crossreact with endoglin expressed on murine cells. This crossreactivity is an unexpected result since (a) it has not been reported previously, and (b) there is significantly less identity in the murine and human sequences encoding the extracellular region (exposed to antibody) of endoglin than that encoding the transmembrane regions and cytoplasmic regions. MAbs K4-2C10, Y4-2F1 and P3-2G8 were generated using the immunization protocol first described in Example 1, whereas D4-2G10 was generated using the second immunization protocol.

The specificity of immunoreactivity of each of these four MAbs was further characterized by testing them against various hematopoietic cell lines in a cellular radioimmunoassay (RIA) and by immunoprecipitation of hEDG using methods previously described (Haruta and Seon, 1986, *supra*). Briefly, 20  $\mu$ l of a 1:9 dilution of the culture fluids of individual hybridomas and  $2 \times 10^5$  hematopoietic cells in each test by RIA. Mouse plasmacytoma IgG1 and IgG2a were included in the assays as controls. The results indicating immunoreactivity (+) or no detectable immunoreactivity (-) are shown in Table 1.

Table 1

Cell line	Origin	K4-2C10	D4-2G10	Y4-2F1	P3-2G8
KM-3	ALL	+	+	+	+
NALM-16	ALL	+	+	+	+
REH	ALL	+	+	ND	ND
NALM-6	ALL	+	+	+	+
NALM-1	CML-BC	+	+	ND	ND
BALL-1	ALL	-	-	-	-

	BALM-2	ALL	-	-	ND	ND
	Daudi	BL	-	-	-	-
	Ramos	BL	-	-	ND	ND
5	U698M	LS	-	-	ND	ND
	BALM-3	LY	-	-	ND	ND
	SU-DHL-4	HL	-	-	ND	ND
	MOLT-4	ALL	-	-	-	-
	JM	ALL	-	-	-	-
10	CCRF-CEM	ALL	-	-	ND	ND
	CCRF-HSB2	ALL	-	-	ND	ND
	Ichikawa	ALL	-	-	ND	ND
	HPB-MLT	LTL	-	-	ND	ND
15	HUT-78	SS	-	-	ND	ND
	HL-60	APL	+	+	+	+
	U937	HL	+	+	+	+
	ML-2	AML	+	+	ND	ND
	CCRF-SB		-	-	-	-
20	RPMI 8057		-	-	ND	ND
	RPMI 1788		-	-	ND	ND

25 ALL- acute lymphoblastic leukemia; CML-BC- chronic myelocytic leukemia in blst crisis; BL- Burkitt's lymphoma; LS- lympho-sarcoma; LY-lymphoma; HL- histiocytic lymphoma; LTL- leukemic phase of T-cell lymphoma; SS- Sezary syndrome; APL- acute pro-myelocytic leukemia; AML, acute myelocytic leukemia; ND- not  
 30 determined.

As shown by Table 1 for the anti-endoglin MAbs of the present invention, and as previously determined for control anti-endoglin MAb SN6, immunoreactivity was  
 35 demonstrated for the immature B-lineage leukemia cell lines tested (KM-3, REH, NALM-1, NALM-6 and NALM-16) and

the myelo-monocytic leukemia cell lines tested (ML-2, HL-60 and U937). However, they did not react with any of the mature B-lineage leukemia-lymphoma cell lines (BALL-1, BALM-2, BALM-3, Daudi, Ramos, U698M, and SU-DHL-4), and the EBV-transformed B cell lines (CCRF-SB, RPMI 1788 and RPMI 8057). The immunoprecipitation assay showed that all four anti-endoglin MAb of the present invention, as did the control anti-hEDG MAb SN6, precipitated a 170 kD component under unreduced conditions and a 92 kD component under reduced conditions.

Using RIA, the 11 anti-hEDG MAb generated using the protocols according to Example 1 and anti-hEDG MAb SN6 were tested for immunoreactivity to proliferating SVEC4-10 murine endothelial cells expressing endoglin at their cell surface. Only the four MAb K4-2C10, D4-2G10, Y4-2F1 and P3-2G8 were found to have binding specificity (at least one standard deviation greater binding than MAb SN6 or control antibody) with the endoglin expressed on the murine endothelial cells. The crossreactivity of these four MAb to murine SVEC4-10 cells is compared with the immunoreactivity of MAb K4-2C10 and D4-2G10 to proliferating human umbilical vein endothelial cells (HUVEC; high expressors of hEDG), at various incubation times in the RIA (2 hours, 4 hours, 8 hours, 24 hours, and 32 hours) as shown in Table 2. The controls are respective species-specific, isotype-matched IgG.

30

Table 2

Cells	MAb	2 hr.	4 hr.	8 hr.	24 hr.	32 hr.
SVEC4 -10	K4-2C10	414 $\pm$ 46	458 $\pm$ 62	698 $\pm$ 44	926 $\pm$ 32	785 $\pm$ 102
	D4-2G10	445 $\pm$ 85	572 $\pm$ 75	1,018 $\pm$ 164	1,222 $\pm$ 62	863 $\pm$ 170
	Y4-2F1	381 $\pm$ 15	ND	ND	758 $\pm$ 64	ND

35

	P3-2G8	394 $\pm$ 36	ND	ND	662 $\pm$ 30	ND
	Control	290 $\pm$ 29	267 $\pm$ 13	463 $\pm$ 58	456 $\pm$ 60	449 $\pm$ 104
5	HUVEC	K4-2C10	8,227 $\pm$ 385	ND	ND	11,346 $\pm$ 1,026
		D4-2G10	7,960 $\pm$ 388	ND	ND	13,077 $\pm$ 876
		Control	196 $\pm$ 24	ND	ND	224 $\pm$ 25

Each test was carried out in triplicate and the values given (as counts per minute) are the mean of triplicates  $\pm$  standard deviation.

10                   The specificity of immunoreactivity of each of the four MAbs was further characterized by using these MAbs in histochemical staining of several human malignant tissues. The tissues included malignant tissues of breast, colon, kidney, lung, and lymph node.

15   The tissues were frozen, then air-dried and fixed with acetone, and stained according to the methods standard in the art. The immunohistochemical staining of the malignant tissue with each of the four MAbs showed that these MAbs reacted strongly with the vascular

20   endothelium associated with all of the malignant tissues tested, whereas the control IgG failed to demonstrate any significant staining in each tissue.

                  In summary, the anti-hEDG MAbs according to the present invention showed strong immunoreactivity to

25   endoglin as expressed by proliferating human vascular endothelial cells, as demonstrated by their reactivity with HUVEC and malignant tumor vasculature. It is important to note that the immunoreactivity was to the vasculature of the malignant tumor tissue, and not to

30   the tumor cells per se. The anti-hEDG MAbs according to the present invention also reacted significantly with the endoglin expressed on proliferating murine endothelial cells, although to a lesser degree as compared to the immunoreactivity with that on HUVEC.

Since endoglin is primarily a proliferation-associated marker for endothelial cells undergoing active angiogenesis, the anti-hEDG MAb according to the present invention may be used to selectively target antiangiogenic therapy to tumor vasculature or the excessive vascularization present in other angiogenesis-associated diseases in both humans and mouse models of human disease.

Any potentially new agents for antiangiogenic therapy need to be evaluated for their safety and efficacy in an animal model before the agents are applied to the clinical trials involving human patients. In this regard, the observed cross-reactivity of the ant-human endoglin MAb according to the present invention with mouse endothelial cells is critically important for evaluating safety and efficacy of these MAb and immunoconjugates formed using these MAb.

### EXAMPLE 3

In this example is illustrated a first embodiment of a method of according to the present invention for targeting therapies against tumor vasculature or the excessive vascularization present in other angiogenesis-associated diseases (collectively referred to as "antiangiogenic therapy"). The method of antiangiogenic therapy according to the present invention is a primary utility for the anti-endoglin MAb according to the present invention. In this embodiment, an immunoconjugate is produced using an anti-endoglin MAb according to the present invention, or a fragment thereof. The anti-endoglin MAb or a fragment thereof is coupled to either at least one antitumor agent, such as an angiogenesis inhibitor, in forming the immunoconjugate. Such immunoconjugate is then used in a relevant mouse model to test the antiangiogenic therapy before scale up to antiangiogenic therapy in humans.

While the immunoconjugate may be administered by routes other than intravenously (i.v.), a preferred embodiment of the illustration is i.v. administration of the immunoconjugate. This is because it is the  
5 proliferating vasculature comprising the angiogenesis that is the target of the therapy; and thus, administering the immunoconjugate i.v. saturates the targeted vasculature much quicker than if another route of administration is used. Additionally, the  
10 intravenous route allows for the possibility of further targeting to specific tissues. Thus, in a variation of this embodiment, a catheter may be used to direct the immunoconjugate to the location of the target angiogenesis. For example, if tumor angiogenesis is the  
15 target of the anti-angiogenic therapy, and if the tumor is located in the liver, then the immunoconjugate may be delivered into the hepatic portal vein using a catheter. In this variation, there is even less systemic distribution of the immunoconjugate, thereby further  
20 minimizing any potential side effects from antiangiogenic therapy.

#### 1. Production of the immunoconjugates

To illustrate this first embodiment, an  
25 immunoconjugate was prepared using each of the four anti-endoglin MABs according to the present invention. The immunoconjugate comprises one of MABs K4-2C10, D4-2G10, Y4-2F1 and P3-2G8 coupled to either ricin A chain ("RA") or deglycosylated ricin A chain ("dgRA") using a  
30 hetero-bifunctional reagent (SMPT) which introduces an in vivo-stable disulfide linker into the IgG molecules of the MAB (see, e.g., Thorpe et al., 1987, Cancer Res. 47:5924-5931). The RA or dgRA is then mixed and incubated with the modified MAB. The resultant MAB-dgRA  
35 conjugates or MAB-RA conjugates are separated from the unbound dgRA or RA, respectively, and then further



purified. A control immunoconjugate is formed in the same manner but with the IgG component being an isotype-matched control IgG (MOPC 195 variant).

5    2. In vitro cytotoxic activity of the immunoconjugates

          The in vitro cytotoxic activity of the various MAb-dgRA conjugates, MAb-RA conjugates, and control conjugate was evaluated by a modified method of May et al. (1990, *J. Immunol.* 144:3637-3642). Briefly,

10    proliferating SVEC4-10 murine endothelial cells, or MCF-7 human breast cancer cells, were distributed into wells of flat-bottomed 96-well microtiter plates at  $2.5 \times 10^4$  cells/well. Varying amounts of the MAb-dgRA conjugates, MAb-RA conjugates, control conjugate, or culture medium

15    (additional control) were added to the wells. The plates were then incubated for 48 hours at 37°C in 5% CO<sub>2</sub>. The cells contained in the wells were then pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H] thymidine for 18 hours, and

20    harvested on glass fiber filters using a semiautomatic cell harvester. The radioactivity was determined using a scintillation counter, and the results were expressed as a percentage of the [<sup>3</sup>H] thymidine incorporated by cells treated with medium only. As shown in FIG. 1A, MAb-RA conjugates (K4-2C10-RA:●; Y4-2F1-RA:▲; & P3-2G8-RA:■)

25    showed significantly more cytotoxicity against the proliferating murine endothelial cells compared to the cytotoxicity by the control-RA conjugate (x). Likewise, and as shown in FIG. 1B, MAb-dgRA conjugates (K4-2C10-dgRA:●; D4-2G10-dgRA:○) showed significantly more

30    cytotoxicity against the proliferating murine endothelial cells compared to the cytotoxicity by the control-dgRA conjugate. The control conjugates showed weak and nonspecific cytotoxic effects when

35    concentrations were higher than 25 nM. Such nonspecific cytotoxic effects at these concentrations have been observed before (see, e.g., Seon, 1984, *Cancer Res.*

44:259-264). However, such nonspecific cytotoxic effects of the control conjugates were found to be insignificant *in vivo*. There was no significant cytotoxicity observed for the MAb-dgRA conjugates, and control conjugates against the MCF-7 human breast cancer cells.

### 3. Antiangiogenic therapy using the immunoconjugates

A MAb-dgRA conjugate, K4-2C10-dgRA, was used to illustrate the antiangiogenic therapy according to the method of the present invention. A MAb-dgRA conjugate was selected for illustrative purposes since conjugates containing dgRA appear more efficacious than conjugates containing RA in *in vivo* tumor therapy when administered *i.v.* (see, e.g. Fulton et al., 1988, *Cancer Res.* 48:2626-2631).

A. Before the immunoconjugate is administered to a mouse model for human disease, a determination should be made of the maximum tolerated dose. In that regard, the LD<sub>50</sub> value of the K4-2C10-dgRA conjugate was determined using a modification of the method described by Fulton et al. (1988, *supra*). Briefly, groups of 4 female BALB/c mice (7 weeks old) were given intraperitoneal injections of the K4-2C10-dgRA conjugate at doses of 0.025, 0.05, 0.1, 0.2, and 0.4 mg. The mice were weighed prior to injection, and daily thereafter, and were observed for morbidity and mortality for 2 weeks. The LD<sub>50</sub> value was determined by plotting the percentage of mortality versus injected dose to determine the dose resulting in 50% mortality. The LD<sub>50</sub> value of the K4-2C10-dgRA conjugate in mice was 16.4 µg/g body weight.

B. SCID mice with human tumor xenografts comprising MCF-7 human breast cancer cells was a model for the evaluation of antiangiogenic therapy comprising administration of K4-2C10-dgRA conjugate. Human breast cancer was used in this model to represent human solid

tumors. To produce this model, MCF-7 cells were transplanted into SCID mice. Preliminary dose-dependent titration experiments showed that all SCID mice inoculated subcutaneously with a dose of  $8 \times 10^6$  cells/mouse developed subcutaneous tumors. Thus,  $8 \times 10^6$  cells was the dosage used to establish subcutaneous tumors in the mice to be treated with the antiangiogenic therapy according to the present invention. Growth of the tumors was monitored daily.

5 C. In a first embodiment and second embodiment, the antiangiogenic therapy comprising administration of K4-2C10-dgRA conjugate was tested in the SCID mouse-MCF-7 xenograft model. Two sets of therapeutic protocols were carried out. In a first therapeutic protocol, a first group of 8 SCID mice that were inoculated subcutaneously with  $8 \times 10^6$  MCF-7 cells/mouse were untreated (control). A second group of 8 SCID mice that were inoculated subcutaneously with  $8 \times 10^6$  MCF-7 cells/mouse were treated by i.v. administration of  $17 \mu\text{g}/0.2 \text{ ml}$  unconjugated (free) MAb K4-2C10. A third group of 8 SCID mice that were inoculated subcutaneously with  $8 \times 10^6$  MCF-7 cells/mouse were treated by i.v. administration with  $20 \mu\text{g}/0.2 \text{ ml}$  K4-2C10-dgRA conjugate. A fourth group of 8 SCID mice that were inoculated subcutaneously with  $8 \times 10^6$  MCF-7 cells/mouse were treated by i.v. administration of unconjugated (free) MAb K4-2C10 ( $75 \mu\text{g}$ ) followed by i.v. administration with the K4-2C10-dgRA conjugate ( $20 \mu\text{g}/0.2 \text{ ml}$ ) on days 3, 5, and 7 post tumor inoculation. Unconjugated MAb K4-2C10, for pretreating the mice comprising group 4, was administered 2 hours post tumor inoculation. The sterilized solutions of the unconjugated MAb K4-2C10, and of the K4-2C10-dgRA conjugate, were diluted with sterile PBS containing mouse serum albumin (0.05% final concentration) before injection via the tail vein of the mice. The total dose of this therapeutic protocol,  $3 \times$

20  $\mu$ g, of the immunoconjugate corresponded to 22% of the LD<sub>50</sub> dose.

During the treatment, the mice were monitored daily for tumor size and for morbidity, and weight of the mice was measured twice each week. Tumor volumes were estimated according to the following equation:

$$V = \text{length} \times (\text{width})^2 \times \pi/6$$

Palpable tumor started appearing 1 to 2 weeks post tumor inoculation for all four groups of mice.

10 However, there were remarkable differences in tumor growth between the immunoconjugate-treated groups and the groups not receiving immunoconjugate treatment. As shown in FIG. 2A, tumors in all of the untreated mice continued to grow for as long as the mice were followed. 15 As shown in FIG. 2B, unconjugated MAb K4-2C10 alone was not significantly effective in inhibiting tumor growth. In contrast, and as shown in FIGS. 2C & 2D, the groups of mice treated with the immunoconjugate (K4-2C10-dgRA conjugate) showed significant tumor regression. Tumors 20 in all eight of the mice treated with immunoconjugate alone (group 3) resulted in complete tumor regression, and the regression continued for as long as the mice were followed (125 days). Thus, based on these results, a first embodiment for effectively delivering 25 antiangiogenic therapy is by administering the immunoconjugate alone. Tumors in seven of eight (87.5%) of the mice pretreated with unconjugated MAb and then treated with the immunoconjugate (group 4) regressed completely, and the regression continued for as long as 30 the mice were followed (125 days post tumor inoculation). Thus, based on these results, a second embodiment for effectively delivering antiangiogenic therapy is by administering unconjugated MAb followed by administering the immunoconjugate. Statistical analysis 35 of the data was carried out using Student's t-test, and Fisher's exact test. In each test, the differences

between the immunoconjugate-treated groups (groups 3 and 4) and the control group (group 1) were statistically significant ( $p < 0.001$ ).

These results indicate that antiangiogenic therapy with the immunoconjugate, comprised of an anti-endoglin MAb according to the present invention and coupled to an antitumor agent, is highly effective in exerting curative antitumor effects in a mouse model of human disease. The antiangiogenic therapy according to either the first or second embodiments of the present invention was effective in inducing complete regression of human solid tumor in the treated SCID mice.

In a second therapeutic protocol, four groups of 6-8 SCID mice inoculated subcutaneously with  $8 \times 10^6$  MCF-7 breast cancer cells/mouse were included. Using the methods according to the first protocol, a first group was untreated (control). A second group was treated by i.v. administration of unconjugated (free) MAb K4-2C10, but on days 3, 4, and 5 post tumor inoculation. A third group was treated by i.v. administration with the K4-2C10-dgRA conjugate, but on days 3, 4, and 5 post tumor inoculation. A fourth group was treated by i.v. administration of unconjugated (free) MAb K4-2C10 (50  $\mu$ g; 1 day post tumor inoculation) followed by i.v. administration with the K4-2C10-dgRA conjugate on days 3, 4, and 5 post tumor inoculation. The mice were evaluated using the methods and parameters according to the first therapeutic protocol.

As shown in FIG. 3A, tumors in all but one of the eight group 1 (untreated) mice continued to grow for as long as the mice were followed. As shown in FIG. 3B, unconjugated MAb K4-2C10 alone was not significantly effective in inhibiting tumor growth. In contrast, and as shown in FIGS. 3C & 3D, the groups of mice treated with the immunoconjugate (K4-2C10-dgRA conjugate) showed significant tumor regression. Tumors in seven of eight

(87.5%) of the mice treated with the immunoconjugate only (group 3) regressed completely, and the regression continued for as long as the mice were followed (105 days post tumor inoculation), as shown in FIG. 3C. As shown in FIG. 3D, tumors in 5 of the 6 mice pretreated with unconjugated anti-endoglin MAb followed by administration of the immunoconjugate (group 4) regressed completely, and the regression continued for as long as the mice were followed (105 days post tumor inoculation). The results of the second therapeutic protocol are highly consistent with the results of the first therapeutic protocol; i.e., antiangiogenic therapy using the anti-endoglin MAb conjugate is effective in inducing lasting complete regression of human solid tumors.

The highly effective *in vivo* antitumor effect of the immunoconjugate is an unexpected result for the following reasons. First, MAb K4-2C10 reacts only weakly with mouse endothelial cells and K4-2C10-dgRA conjugate is only weakly cytotoxic to mouse endothelial cells (as compared to proliferating human endothelial cells). Furthermore, a relatively small amount (corresponding to 22% of LD<sub>50</sub> dose) of the immunoconjugate was administered *i.v.* In the conventional tumor-cell targeted therapy, even highly potent antitumor immunotoxins have rarely shown such strong *in vivo* antitumor effects when a small amount of the agent was administered *i.v.* As taught in the present invention, the immunoconjugate is selectively targeted to vasculature rather than being directed to the tumor *per se*. However, unlike other reported attempts of using targeting agents against tumor angiogenesis (as reviewed in Folkman, 1995, *supra*; Sipos et al., 1994, *Ann. NY Acad. Sci.* 732:263-272; Hawkins, 1995, *Curr. Opin. Oncol.* 7:90-93), using anti-human endoglin MAb in immunoconjugates according to the

present invention appears able to exert curative antitumor effects.

#### EXAMPLE 4

5                   In Example 3 was illustrated a first embodiment and second embodiment of a method of according to the present invention for targeting therapies against tumor vasculature or the excessive vascularization present in angiogenesis-associated  
10 diseases. Using the method according to the first embodiment, it was demonstrated that an immunoconjugate comprising an anti-endoglin MAb according to the present invention and coupled to an antitumor agent will destroy tumor-associated vasculature by selectively reacting  
15 with proliferating endothelial cells of tumor-associated neovasculature and destroying existing tumor-associated blood vessels, thereby preventing tumor-associated angiogenesis from developing (see, e.g., FIGS. 2C and 3C). In Example 3 is illustrated a second embodiment of  
20 a method of according to the present invention for antiangiogenic therapy.

                  According to the second embodiment, utilized is an immunoconjugate produced using an anti-endoglin MAb according to the present invention, or a fragment  
25 thereof, coupled to either at least one antitumor agent, or an angiogenesis inhibitor in forming the immunoconjugate. However, in this second embodiment of antiangiogenic therapy, the tumor-bearing host or the host having excessive vascularization due to an  
30 angiogenesis-associated disease is pretreated with unconjugated anti-endoglin MAb according to the present invention, or a fragment thereof (e.g. F(ab')<sub>2</sub>). The rationale for pretreatment prior to administration of the immunoconjugate is that the pretreatment with an  
35 unconjugated anti-endoglin MAb recognizing the same epitope on the endoglin as the immunoconjugate may

further enhance the selectivity of the immunoconjugate for the target vasculature. For example, endothelial cell turnover in normal quiescent vasculature is very slow. In contrast, the endothelial cells found in the tumor-associated neovasculature or in the excessive vascularization present in angiogenesis-associated diseases undergo rapid proliferation during spurts of angiogenesis (see, e.g. Folkman et al., 1992, *J. Biol. Chem.* 267:10931-10934). Therefore, pretreatment of the host with unconjugated anti-endoglin MAb, or a fragment thereof, may be able to mask (precoat) the weak binding sites on quiescent endothelial cells in the normal (undiseased) tissues, while high amounts of newly generated binding sites on the proliferating endothelial cells found in angiogenesis are then available for binding by a subsequent administration of the immunoconjugate. Precoating normal tissue before administration of the immunoconjugate may reduce undesirable side effects of the administered immunoconjugate potentially caused by the immunoconjugate reacting weakly with normal tissue. Additionally, the precoating step may enhance the antiangiogenic efficacy of the immunoconjugate because of the more efficient delivery of the systemically administered immunoconjugate to the target vasculature. As illustrated in FIGs. 2D and 3D, vasculature (tumor-associated neovasculature, or excessive vascularization present in angiogenesis-associated diseases) can be effectively targeted by systemic administration of the immunoconjugate after the host was administered a precoating of unconjugated anti-endoglin MAb. Thus, i.v. administration of unconjugated anti-endoglin MAb into patients as a precoating step, followed by i.v. administration with anti-endoglin immunoconjugate, is a novel and effective approach to antiangiogenic therapy.



**EXAMPLE 5**

Illustrated and detailed in Examples 3 and 4 were two embodiments for a method according to the present invention for targeting therapies against tumor vasculature, or the excessive vascularization present in angiogenesis-associated diseases, in the SCID mouse-human xenograft model. Another mouse model of human angiogenesis-associated disease was utilized to demonstrate the antiangiogenic therapy using the anti-endoglin MAb and methods of use according to the present invention. Antiangiogenic activity of the immunoconjugate, K4-2C10-dgRA, was tested by the dorsal air sac method (see, e.g., Asano et al., 1995, *Cancer Res.* 55: 5296-5301), an assay for *in vivo* angiogenesis. Briefly,  $1 \times 10^7$  HT1080 human tumor cells were suspended in PBS and then placed into a plastic chamber (14 mm in diameter) which was sealed at each chamber end with a cellulose membrane filter (0.45  $\mu$ m pore size). A chamber, so prepared, was implanted into a dorsal air sac of each of several female BALB/c mice. One group of mice containing the implants was untreated as a positive control of angiogenesis. A second group of mice containing the implants was treated by i.v. administration with unconjugated MAb K4-2C10 (17  $\mu$ g/0.2 ml) 2 hours, 24 hours and 48 hours post chamber implantation. A third group of mice containing the implants was treated by i.v. administration with immunoconjugate, K4-2C10-dgRA (20  $\mu$ g/0.2 ml) 2 hours, 24 hours and 48 hours post chamber implantation. A fourth group of mice was implanted with chambers only containing PBS (without any tumor cells) and untreated as a negative control of angiogenesis. On day 4, the mice of each group were analyzed for the formation of new blood vessels (angiogenesis) in subcutaneous regions. Treatment of mice with the immunoconjugate resulted in significant suppression of angiogenesis,

whereas mice treated with unconjugated MAb K4-2C10 showed much less inhibition of angiogenesis. These results further support the administration of anti-endoglin immunoconjugate as a novel and effective approach to antiangiogenic therapy.

#### EXAMPLE 6

In Examples 3 and 4 were illustrated in mouse models of human disease, two embodiments for a method of according to the present invention for targeting therapies against tumor vasculature or the excessive vascularization present in angiogenesis-associated diseases. Using the method according to either embodiment, it was demonstrated that an immunoconjugate comprising an anti-endoglin MAb according to the present invention and coupled to an antitumor agent will selectively react with, and destroy, proliferating endothelial cells of the targeted vasculature, thereby preventing the associated angiogenesis from developing. Further, precoating with unconjugated anti-endoglin MAb followed by administration of the immunoconjugate is also effective in antiangiogenic therapy.

One skilled in the art would appreciate that the antiangiogenic therapy according to the first or second embodiments would need to be optimized for clinical use in humans. A physiological basis for scaling up the therapeutic agents comprising MAbs from a mouse model to humans is known to those skilled in the art (see, e.g., Baxter et al., 1995, *supra*). For example, the optimal timing for the subsequent administration of immunoconjugate after pretreatment with unconjugated anti-endoglin MAb, and the optimal molar ratio of unconjugated anti-endoglin MAb to immunoconjugate, may need to be determined for individual patients to be treated for tumor-associated neovasculature or the excessive vascularization present

in angiogenesis-associated diseases. The optimal timing will be influenced by several factors such as the pharmacokinetics of the administered anti-endoglin MAb according to the present invention, or a fragment thereof. Also a factor is the rate of endocytosis/shedding of the MAb, or fragment thereof, bound to human endoglin on the surface of human endothelial cells. Pretreatment of patients with autologous or homologous anti-endoglin MAb may enhance the safety and efficacy of the clinical application of an anti-hEDG MAb immunoconjugate according to the method of the present invention.

In another embodiment of the antiangiogenic therapy according to the present invention, a human patient having an angiogenesis-associated disease such as diabetic retinopathy, chronic inflammatory diseases, rheumatoid arthritis, dermatitis, or psoriasis is treated with a therapeutic protocol of multiple administrations of an immunoconjugate according to the present invention. The immunoconjugate comprises an anti-human endoglin MAb according to the present invention, or a fragment thereof, which is coupled to at least one angiogenesis inhibitor or to at least one antitumor agent. The immunoconjugate may further comprise a pharmaceutically acceptable carrier medium. In a further embodiment, the human patient is pretreated with unconjugated anti-endoglin MAb (or a fragment thereof) followed by administration of an anti-hEDG MAb (or a fragment thereof) immunoconjugate according to the present invention. The unconjugated anti-endoglin MAb (or a fragment thereof) may further comprise a pharmaceutically acceptable carrier medium.

In a further embodiment of the antiangiogenic therapy according to the present invention, a human patient having a tumor such as a human leukemia, including non-T-cell-type (non-T) acute lymphoblastic

leukemia (ALL), myelo-monocytic leukemia; or a human solid tumor, including angiosarcoma, breast carcinoma, cecum carcinoma, colon carcinoma, Hodgkins lymphoma, lymphoma, lung carcinoma, melanoma, osteosarcoma, ovarian carcinoma, parotid tumor, pharyngeal carcinoma, and rectosigmoid carcinoma, is treated with a therapeutic protocol of multiple administrations of an immunoconjugate according to the present invention. The immunoconjugate comprises an anti-human endoglin MAb according to the present invention, or a fragment thereof, which is coupled to at least one angiogenesis inhibitor or to at least one antitumor agent. The immunoconjugate may further comprise a pharmaceutically acceptable carrier medium. In a further embodiment, the human patient is pretreated with unconjugated anti-endoglin MAb (or a fragment thereof) followed by administration of an anti-hEDG MAb (or a fragment thereof) immunoconjugate according the present invention. The unconjugated anti-endoglin MAb (or a fragment thereof) may further comprise a pharmaceutically acceptable carrier medium.

One of skill in the art would appreciate that depending on the angiogenesis inhibitor or antitumor agent used, the therapeutic protocol may require additional steps. For example, where the administered immunoconjugate comprises an anti-hEDG (or fragment thereof) conjugated to a photosensitizer (mostly porphyrins), a further step of irradiation with light of the targeted area is required.

30

#### EXAMPLE 7

In Examples 3-5 was demonstrated the administration of anti-endoglin immunoconjugate as a novel and effective approach to antiangiogenic therapy, wherein the anti-endoglin MAb is used to target therapies against tumor vasculature or the excessive

35

vascularization present in angiogenesis-associated diseases. While the monoclonal antibody used to illustrate the effectiveness of antiangiogenic therapy according to the method of the present invention is a murine monoclonal antibody, it will be appreciated by those skilled in the art that such murine MABs may be modified ("engineered") using techniques standard in the art (e.g., as reviewed by Adair, 1992, *Immunological Reviews* 130: 6-37, herein incorporated by reference).

For example, murine monoclonal antibodies may be "humanized" by replacing portions of the murine monoclonal antibody with the equivalent human sequence. In one embodiment, a chimeric antibody is constructed. The construction of chimeric antibodies is now a straightforward procedure (Adair, 1992, *supra*, at p. 13) in which the chimeric antibody is made by joining the murine variable region to a human constant region. Additionally, chimeric antibodies may be made by joining the hypervariable regions of the murine monoclonal antibody to human constant regions and parts of human variable regions using one of several techniques known in the art. Techniques for constructing chimeric antibodies (murine-human) of therapeutic potential have been described previously (see, e.g., Morrison et al., 1984, *Proc. Natl. Acad. Sci.* 81:6851-6855; Larrick et al., 1991, *Hum. Antibod. Hybridomas* 2:172-189; herein incorporated by reference). Thus, in one embodiment of the present invention, and using methods known in the art, the murine variable region of the anti-endoglin MAB according to the present invention is joined to a human constant region to form a chimeric anti-endoglin antibody having the same specificity as the anti-endoglin MAB. In general, humanizing an murine MAB such as by making a chimeric antibody limits the development of human anti-mouse antibody responses. Additionally, the humanized antibodies generally change the

pharmacokinetics by providing a longer half-life of immunoconjugates containing such antibody, as compared to the half-life of immunoconjugates containing murine antibody.

5           A chimeric MAb can also be constructed using a standard combination of techniques including polymerase chain reaction (PCR) cloning of antibody variable regions, the use of suitable expression vectors already containing the DNA encoding human constant region,  
10   insertion of the DNA for the murine MAb variable region into such vector in forming a recombinant vector, and expression of the resultant chimeric antibody by an expression system containing the recombinant vector (See, e.g., Daugherty et al., 1991, *Nucl. Acids Res.*  
15   19:2471-2476; Maeda et al., 1991, *Human Antibodies and Hybridomas* 2:124-134; herein incorporated by reference). One expression vector can be used in which the vector is constructed so that the variable region and constant region genes are in tandem. Alternatively, the DNA  
20   encoding the mouse variable region is inserted into one expression vector, and the DNA encoding the human constant region can be inserted into a second expression vector, followed by transfections using both the first and second expression vectors. Expression systems known  
25   to those skilled in the art for production of antibody or antibody fragments include mammalian cells (e.g. cell lines such as COS, NSO, or CHO), phage expression libraries, *Escherichia coli*, and yeast (Adair, 1992, *supra*).

30

From the foregoing, it will be obvious to those skilled in the art that various modifications in the above-described methods, and compositions can be made without departing from the spirit and scope of the  
35   invention. Accordingly, the invention may be embodied in other specific forms without departing from the

spirit or essential characteristics thereof. Present  
embodiments and examples, therefore, are to be  
considered in all respects as illustrative and not  
restrictive, and all changes which come within the  
5 meaning and range of equivalency of the claims are  
therefore intended to be embraced therein.

What is claimed is:

1. A monoclonal antibody, or fragments thereof, having detectable binding specificity for crossreactive  
5 epitopes shared by endoglin expressed on proliferating human vascular endothelial cells and endoglin expressed on proliferating murine endothelial cells, wherein the binding to endoglin expressed on proliferating murine endothelial cells is of a lesser degree as compared to  
10 the binding of endoglin expressed on proliferating human vascular endothelial cells.
2. Fragments of the monoclonal antibody according to claim 1, wherein the fragments are selected from the  
15 group consisting of F(ab')<sub>2</sub>, Fab', Fab, Fv, Fd' and Fd.
3. The monoclonal antibody according to claim 1, wherein the antibody is a chimeric antibody comprising a murine variable region having anti-endoglin binding  
20 specificity for crossreactive epitopes shared by human endoglin and murine endoglin, and a human constant region.
4. A composition for antiangiogenic therapeutic  
25 treatment of an angiogenesis-associated disease comprising tumor angiogenesis or other angiogenesis-associated disease in a mammalian host, wherein the composition comprises an immunoconjugate formed from a therapeutically effective amount of the monoclonal  
30 antibody, or fragments thereof, according to claim 1 conjugated to a therapeutically effective amount of an agent selected from the group consisting of at least one angiogenesis inhibitor and at least one antitumor agent.
- 35 5. The composition for antiangiogenic therapeutic treatment according to claim 4, wherein the fragments



- 39 -

are selected from the group consisting of  $F(ab')_2$ ,  $Fab'$ ,  $Fab$ ,  $Fv$ ,  $Fd'$  and  $Fd$ .

6. The composition for antiangiogenic therapeutic  
5 treatment according to claim 4, wherein the antibody is  
a chimeric antibody comprising a murine variable region  
having anti-endoglin binding specificity for  
crossreactive epitopes between human endoglin and murine  
endoglin, and a human constant region.
- 10 7. The composition for antiangiogenic therapeutic  
treatment according to claim 4, further comprising a  
pharmaceutically acceptable carrier medium for the  
immunoconjugate.
- 15 8. The composition for antiangiogenic therapeutic  
treatment according to claim 4, wherein the treatment is  
of tumor angiogenesis.
- 20 9. The composition for antiangiogenic therapeutic  
treatment according to claim 5, wherein the treatment is  
of tumor angiogenesis.
- 25 10. The composition for antiangiogenic therapeutic  
treatment according to claim 6, wherein the treatment is  
of tumor angiogenesis.
- 30 11. The composition for antiangiogenic therapeutic  
treatment according to claim 8, wherein the agent is an  
antitumor agent selected from the group consisting of  
ricin A chain and deglycosylated ricin A chain.
- 35 12. The composition for antiangiogenic therapeutic  
treatment according to claim 9, wherein the agent is an  
antitumor agent selected from the group consisting of  
ricin A chain and deglycosylated ricin A chain.

13. The composition for antiangiogenic therapeutic treatment according to claim 10, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.
- 5
14. The composition for antiangiogenic therapeutic treatment according to claim 4, wherein the treatment is of an angiogenesis-associated disease other than tumor.
- 10
15. The composition for antiangiogenic therapeutic treatment according to claim 5, wherein the treatment is of an angiogenesis-associated disease other than tumor.
- 15
16. The composition for antiangiogenic therapeutic treatment according to claim 6, wherein the treatment is of an angiogenesis-associated disease other than tumor.
- 20
17. The composition for antiangiogenic therapeutic treatment according to claim 14, wherein the agent is an angiogenesis inhibitor.
- 25
18. The composition for antiangiogenic therapeutic treatment according to claim 15, wherein the agent is an angiogenesis inhibitor.
- 30
20. A method for antiangiogenic therapy of tumor in a mammalian host comprising administering a therapeutically effective amount of a composition comprising the immunoconjugate according to claim 4.
- 35
21. A method for antiangiogenic therapy of tumor in a mammalian host comprising administering a

therapeutically effective amount of a composition comprising the immunoconjugate according to claim 5.

22. A method for antiangiogenic therapy of tumor in a mammalian host comprising administering a therapeutically effective amount of a composition comprising the immunoconjugate according to claim 6.

23. The method for antiangiogenic therapy according to claim 20, further comprising administering a pharmaceutically acceptable carrier medium with the immunoconjugate.

24. The method for antiangiogenic therapy according to claim 21, further comprising administering a pharmaceutically acceptable carrier medium with the immunoconjugate.

25. The method for antiangiogenic therapy according to claim 22, further comprising administering a pharmaceutically acceptable carrier medium with the immunoconjugate.

26. The method for antiangiogenic therapy according to claim 20, wherein the composition is administered intravenously.

27. The method for antiangiogenic therapy according to claim 21, wherein the composition is administered intravenously.

28. The method for antiangiogenic therapy according to claim 22, wherein the composition is administered intravenously.

29. The method for antiangiogenic therapy according to claim 20, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

5

30. The method for antiangiogenic therapy according to claim 21, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

10

31. The method for antiangiogenic therapy according to claim 22, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

15

32. A method for antiangiogenic therapy of an angiogenesis- associated disease comprising tumor in a mammalian host, said method comprising administering in sequential order a therapeutically effective amount of:

20

(a) the monoclonal antibody, or fragments thereof, according to claim 1 in an unconjugated form; and

25

(b) a composition comprising the monoclonal antibody, or fragments thereof, according to claim 1 conjugated to an agent selected from the group consisting of at least one angiogenesis inhibitor and at least one antitumor agent in forming an immunoconjugate;

30

wherein pretreatment with the unconjugated monoclonal antibody, or a fragment thereof, precoats weak binding sites on quiescent vascular endothelial cells in normal tissues of the host.

33. The method for antiangiogenic therapy according to claim 32, wherein the fragments are selected from the group consisting of  $F(ab')_2$ ,  $Fab'$ ,  $Fab$ ,  $Fv$ ,  $Fd'$  and  $Fd$ .

35

34. The method for antiangiogenic therapy according to claim 32, wherein the antibody is a chimeric antibody comprising a murine variable region having anti-endoglin binding specificity for crossreactive epitopes shared by human endoglin and murine endoglin, and a human constant region.

35. The method for antiangiogenic therapy according to claim 32, further comprising administering a pharmaceutically acceptable carrier medium with the unconjugated monoclonal antibody, or a fragment thereof, and with the immunoconjugate.

36. The method for antiangiogenic therapy according to claim 32, wherein the unconjugated monoclonal antibody, or a fragment thereof, and the immunoconjugate are administered intravenously.

37. The method for antiangiogenic therapy according to claim 32, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

38. The method for antiangiogenic therapy according to claim 33, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

39. The method for antiangiogenic therapy according to claim 34, wherein the agent is an antitumor agent selected from the group consisting of ricin A chain and deglycosylated ricin A chain.

40. A method for antiangiogenic therapy of angiogenesis-associated disease other than tumor in a

mammalian host comprising administering in sequential order a therapeutically effective amount of:

(a) the monoclonal antibody, or fragments thereof, according to claim 1 in an unconjugated form; and

5 (b) a composition comprising the monoclonal antibody, or fragments thereof, according to claim 1 conjugated to at least one angiogenesis inhibitor in forming an immunoconjugate;

10 wherein pretreatment with the unconjugated monoclonal antibody, or a fragment thereof, precoats weak binding sites on quiescent vascular endothelial cells in normal tissues of the host.

41. The method for antiangiogenic therapy according to  
15 claim 40, wherein the fragments are selected from the group consisting of F(ab')<sub>2</sub>, Fab', Fab, Fv, Fd' and Fd.

42. The method for antiangiogenic therapy according to  
20 claim 40, wherein the antibody is a chimeric antibody comprising a murine variable region having anti-endoglin binding specificity for crossreactive epitopes shared by human endoglin and murine endoglin, and a human constant region.

25 43. The method for antiangiogenic therapy according to claim 40, further comprising administering a pharmaceutically acceptable carrier medium with the unconjugated monoclonal antibody, or a fragment thereof, and with the immunoconjugate.

30 44. The method for antiangiogenic therapy according to claim 40, wherein the unconjugated monoclonal antibody, or a fragment thereof, and the immunoconjugate are administered intravenously.

35

FIG. 1A

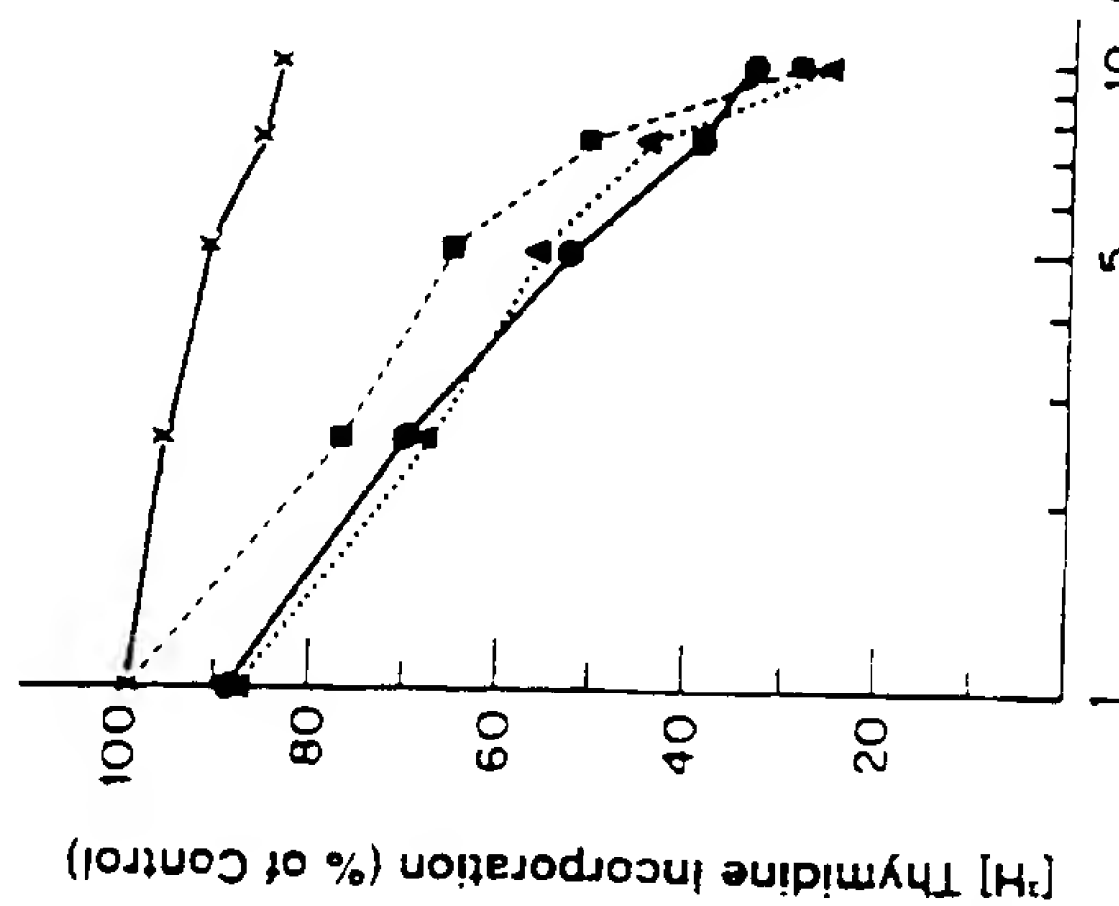
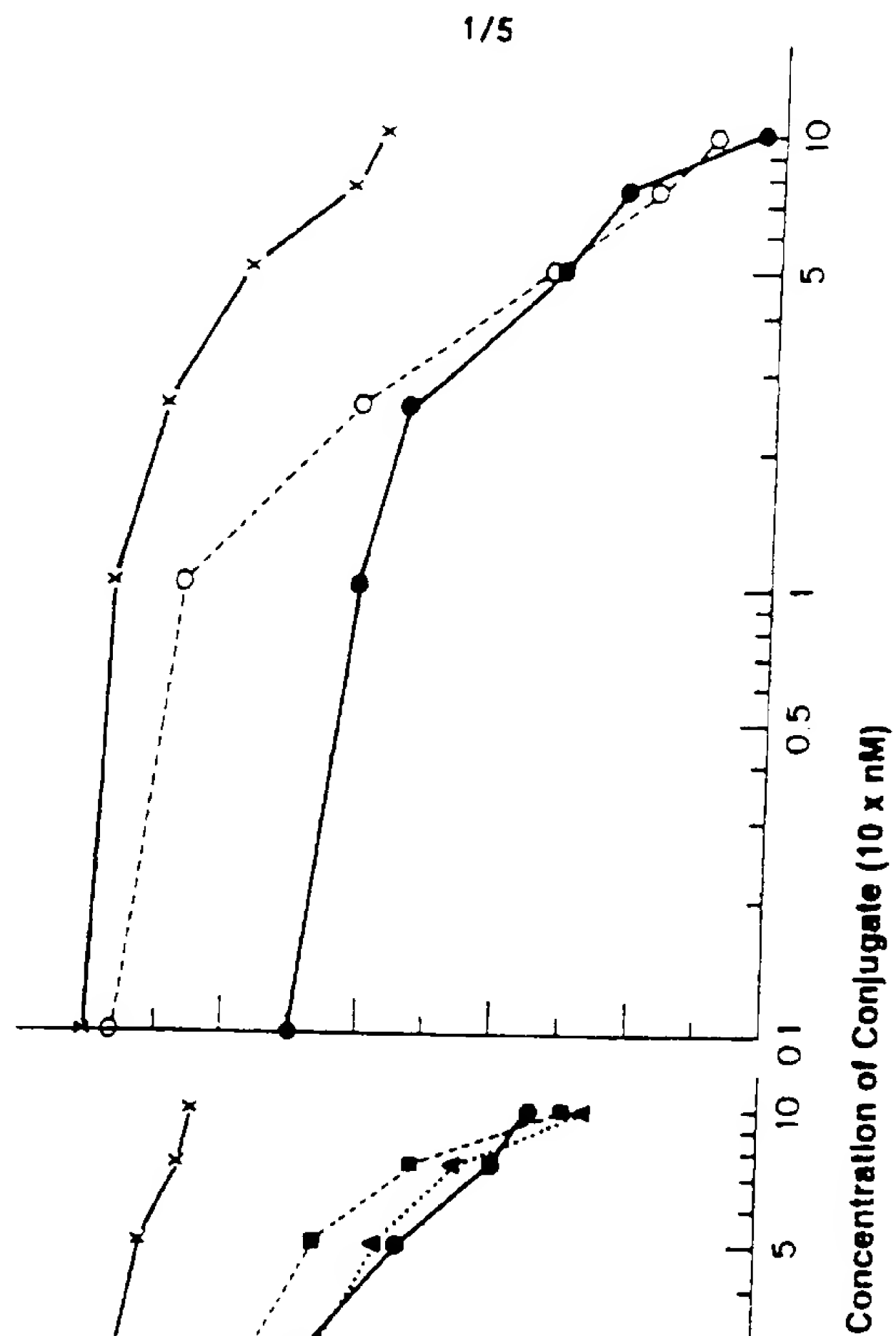
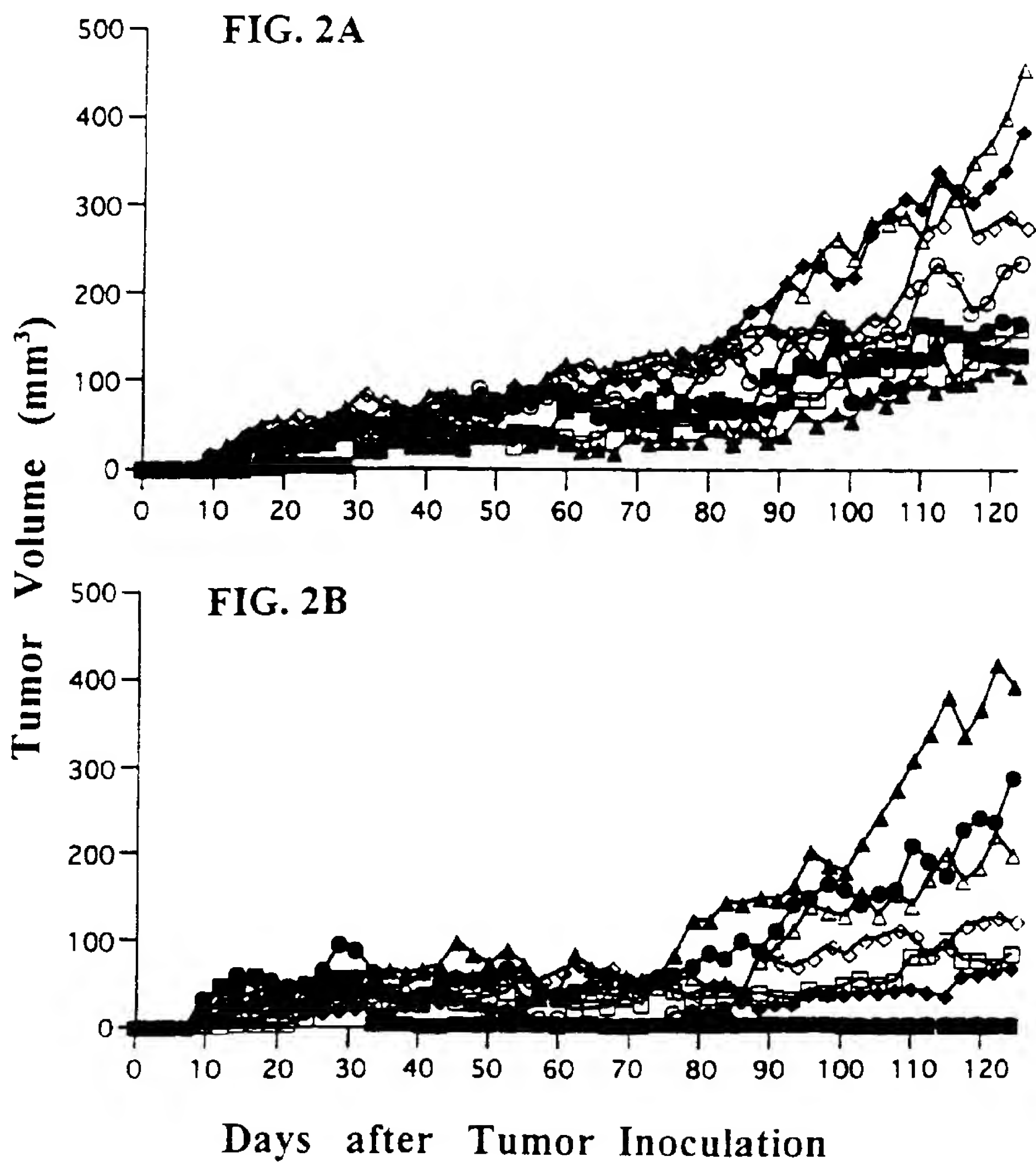


FIG. 1B



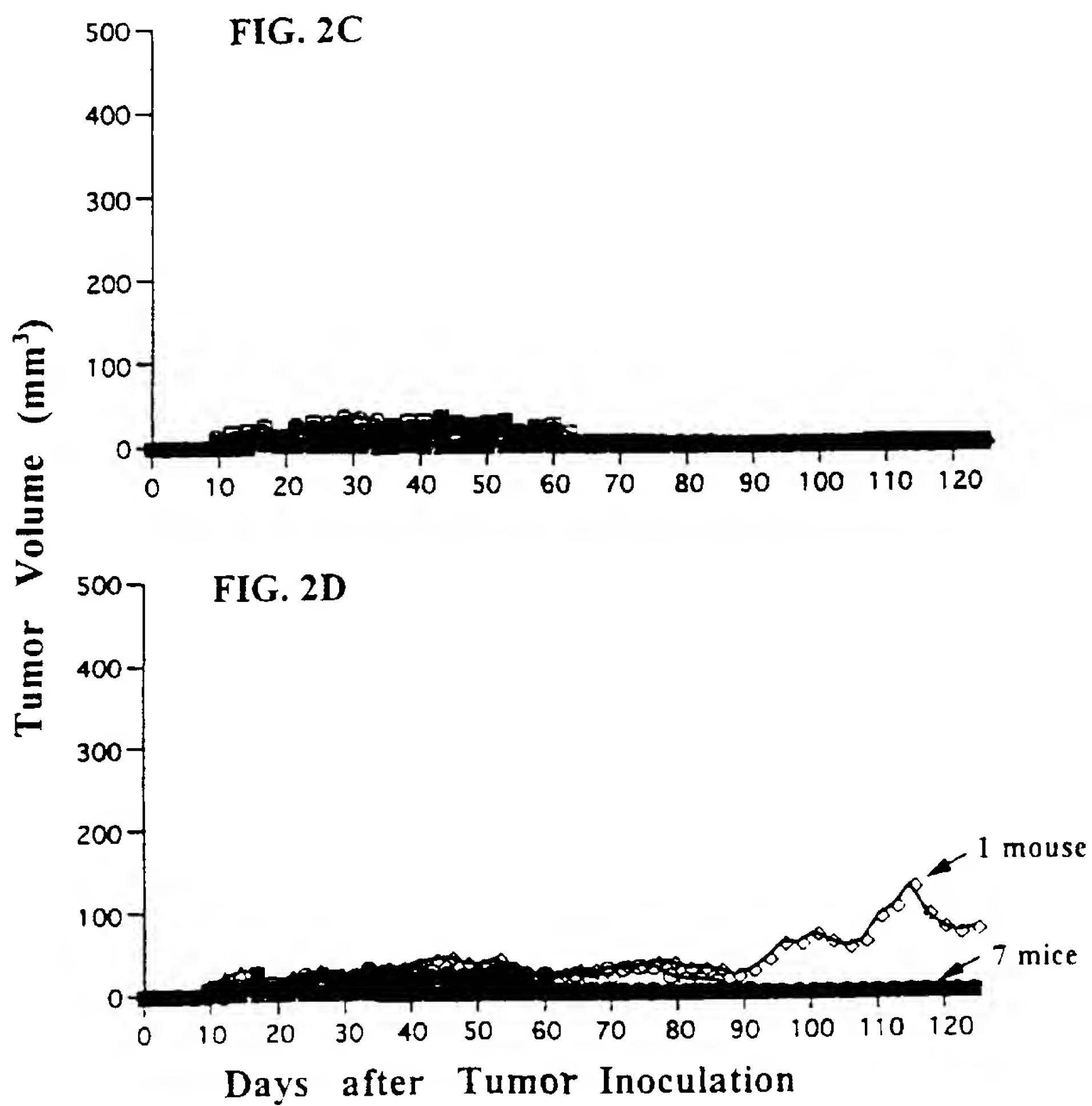
1/5

2/5

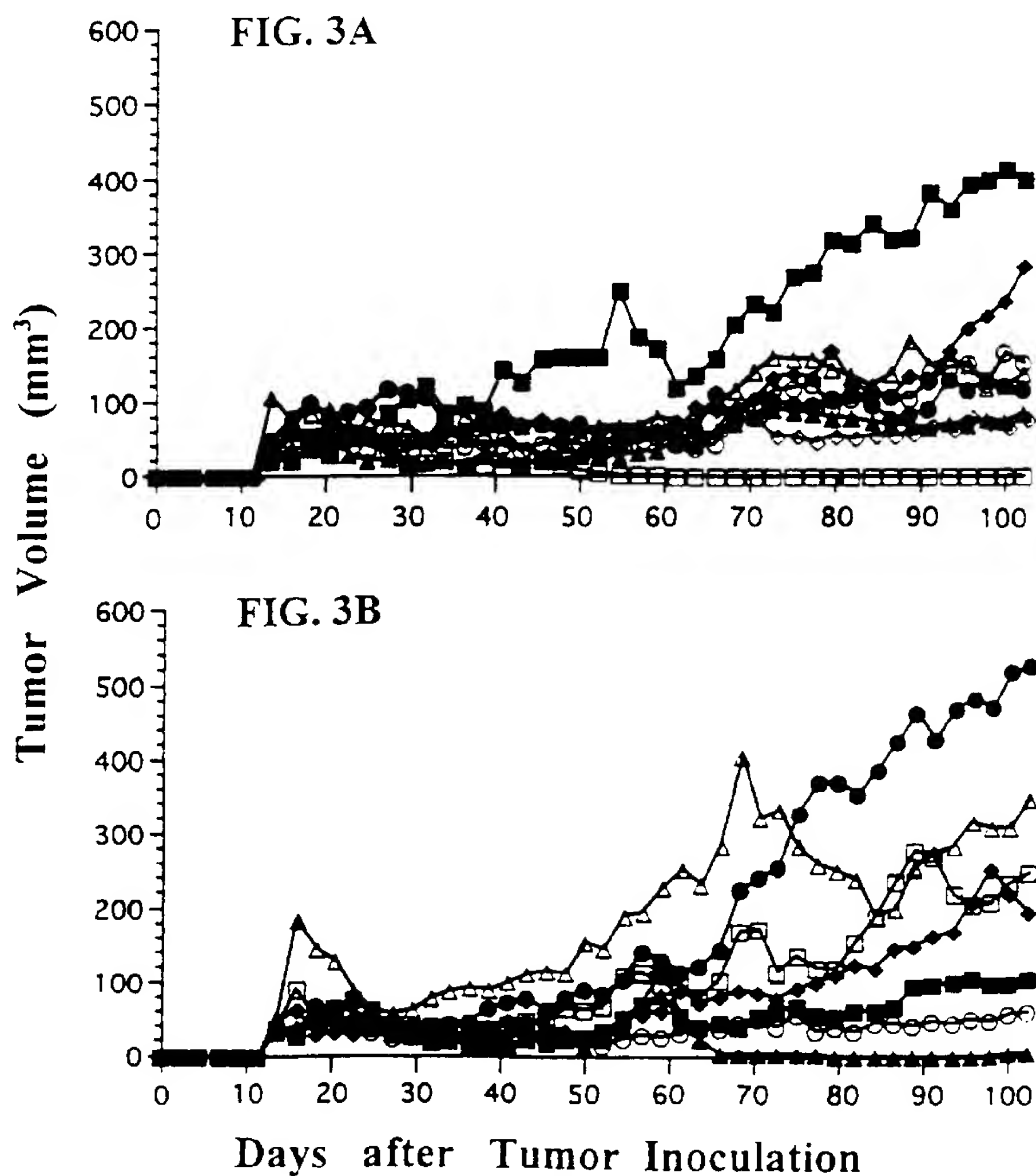




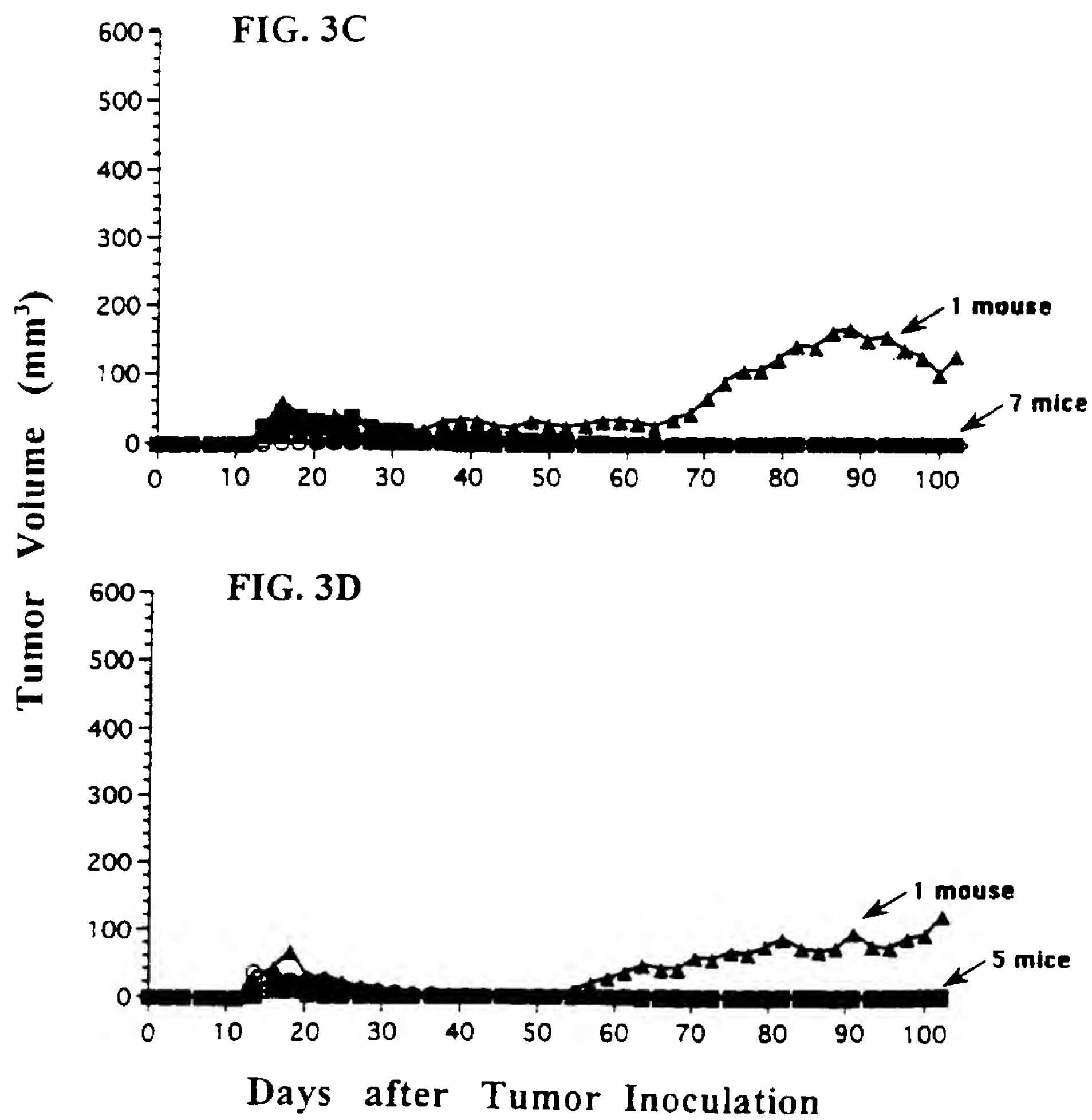
3/5



4/5



5/5



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/09610

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 16/00; C12P 21/08; A61K 39/395

US CL : 424/130.1, 156.1; 530/388.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 156.1; 530/388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

dialog, APS, medline search terms: endoglin, antiangiogenesis, antibody(s), conjugate(s), toxin(s) immunotoxin(s)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BETTER, M. et al. Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab' and F(ab') <sub>2</sub> . Proc. Natl. Acad. Sci. USA. 1993, Vol. 90, pages 457-461, see entire document.	1, 4-10, 12-19, 22, 25, 28, 31, 34, 39, 42
X	GE, A. Z. et al. Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF- $\beta$ ligand. Gene. 1994, pages 201-206, see entire document.	1
X	HARUTA, Y. et al. Distinct human leukemia-associated cell surface glycoprotein GP160 defined by monoclonal antibody SN6. Proc. Natl. Acad. Sci. USA. October 1986, Vol. 83, pages 7898-7902, see entire document.	1

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to underpin the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

21 JULY 1997

Date of mailing of the international search report

13 AUG 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

NEAL A. MUSTO, PH.D.

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/09610

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OWENS, R. J. et al. The genetic engineering of monoclonal antibodies. J. Immunol. Methods. 1994, Vol. 168, pages 149-165, see entire document.	1-3, 5, 21, 24, 27, 30, 33, 38, 41
Y	SEVIER, E. D. et al. Monoclonal antibodies in clinical immunology. Clin. Chem. 1981, Vol. 27, No. 11, pages 1797-1805, see entire document.	1-20, 23, 26, 29, 32-44
Y	ST-JACQUES, S. et al. Molecular characterization and <i>in situ</i> localization of murine endoglin reveal that it is a transforming growth factor- $\beta$ binding protein of endothelial and stromal cells. Endocrinology. 1994, Vol. 134, No. 6, pages 2645-2657, see entire document.	1-19
Y	THORPE, P. E. et al. Antibody-directed targeting of the vasculature of solid tumors. Breast Cancer Research Treatment. 1995, Vol. 36, pages 237-251, see entire document.	1, 4-44